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An investigation of the prevalence of *Giardia agilis* in anuran amphibians from fourteen areas in China

Zhangxia Lyu[a,b,1], Jiaoni Cheng[a,1], Jingru Shao[a], Qingqing Ye[a], Huixian Bai[a], Jianfan Wen[a,*]

[a] State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China
[b] Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, 650204, China

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ABSTRACT

Giardia agilis is a *Giardia* species which is morphological distinguishable for its very narrow and elongated trophozoite. Although there were a few studies about its morphology since its first report in 1882, none investigations about its prevalence have ever been reported to date. We investigated the prevalence of *G. agilis* in 25 anuran amphibian species from five provinces of China using both morphological and molecular methods. Of the 463 tested samples, 195 (42.1%) were positive. The 195 positive samples were from nine species, which are scatteredly distributed in four anuran amphibian families. The statistical prevalence among adults of different frog species showed no significant difference, and so did among tadpoles. Thus, *G. agilis* is probably able to infect all anuran amphibians without species-bias. More interestingly, the prevalence in the tadpoles is significantly higher than in their adults. The prevalence in *Kaloula verrucosa* tadpoles from the same area showed no significant differences between none-legged stage and two-legged stage, but the prevalence in these two developmental stages is significantly higher than in the four-legged stage. And the prevalence in four-legged stage is still much higher than in adults. A turning point of prevalence appeared in the period of tadpole tail degeneration. Moreover, all the positive samples were from the areas with relatively high altitude (more than 870 m). The fact that *G. agilis* tends to easily infect the frogs living in high altitude areas indicated it has evolved the ability to adapted the dramatic temperature change in poikilothermal animals. Therefore, *G. agilis* has evolved some special successful parasitism strategies for parasitizing the poikilothermal hosts with metamorphosis such as anuran amphibians.

1. Background

Giardia are intestinal protozoan parasites found in almost all vertebrates worldwide, and have attracted the attention of scientists for both medical and evolutionary biological reasons in the past 300 years (Adam, 2001). There are currently eight *Giardia* species considered to be valid (Lyu et al., 2018). *Giardia agilis*, as one of them, was first reported by Künstler in 1882, and was later found in the intestines of tadpoles and adults of several anuran amphibian species by other researchers (Adam, 2001; Sogayar and Gregório, 1998). The morphologies of *G. agilis* have been studied based on interference reflexion microscopy and scanning electron microscopy (SEM) (Feely and Erlandsen, 1985; Sogayar and Gregório, 1998). The trophozoites of *G. agilis* have a narrow and elongated body, and the length of their adhesive discs is about one fifth of their body (Feely and Erlandsen, 1985). Thus, it can be easily distinguished from other *Giardia* species for its distinctly morphological characteristics. Currently, molecular identification of *G. intestinalis* (Syn. *G. duodenalis* or *G. lamblia*) is usually based on the following five genes: *glutamate dehydrogenase*, *beta-giardin*, *elongation factor-1 alpha*, *triose phosphate isomerase* and *small subunit rRNA* (*SSU rRNA*) (Cacciò and Ryan, 2008). Except *beta-giardin*, all other genes of *G. agilis* have not been sequenced yet (Lyu et al., 2018).

Investigations of the prevalence of *Giardia* have been carried out mainly on *G. intestinalis*, and more than 100 waterborne giardiasis outbreaks had been reported worldwide till 2004 (Karanis et al., 2007). Potential mechanisms of transmission were one of the important contents of the investigations, including person to person, animal to animal and zoonotic through drinking water, recreational contact (such as swimming) and foodborne (Karanis et al., 2007; Shields et al., 2008; Takizawa and Falavigna, 2009). The host specificity of different *G. intestinalis* genotypes was another important content studied, and more than 50 different mammal hosts were investigated including wild

* Corresponding author.
E-mail address: wenjf@mail.kiz.ac.cn (J. Wen).
[1] Equal contributors.

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animals, pets and livestock (Plutzer et al., 2010; Lujan and Svard, 2011). Epidemiology of *G. intestinalis* has been a continuous and hot research topic in the protozoan parasite field due to the harm of giardiasis to human and other mammals.

Unfortunately, we know almost nothing about the prevalence of *G. agilis* although this species has been reported more than one hundred years. There are ten anuran amphibian families containing 386 species in China. In the present study, the prevalence of *G. agilis* was investigated in several anuran amphibians in five provinces of China including Yunnan, Sichuan, Shaanxi, Gansu and Shanghai. We also sequenced their *SSU rRNA* and *beta-giardin* genes to access the molecular identification and phylogenetic analysis. We also compared the results from different developing stages of the same species, and compared the results between different study sites. The possible reasons and implications of these observations are discussed.

2. Material and method

2.1. Sample collection

All anuran amphibian samples were captured by landing net and were unselected. The anuran amphibian species were identified according to a guide book (Atlas of Amphibians of China, Fei Liang et al., 1999) and with the help of amphibian taxonomists. The anuran amphibian samples were transported to lab by tanks (two adults or 10 tadpoles per tank, 40 cm * 30 cm * 25 cm). And the anuran amphibian samples were kept in tanks for 12 h before euthanizing to collect feces samples. *Giardia agilis* trophozoites were collected from frogs and tadpoles using the methodology adapted from Lyu et al. (2018). The frogs and tadpoles were anesthetized using MS-222. Then their intestines were removed and cut into 0.1 cm segments. The segments of intestines were transferred into centrifuge tubes with 0.65% sodium chloride solution. The centrifuge tubes were chilled in ice for more than 30 min. The suspension was briefly centrifuged at 1,000× g for 1s to remove the precipitants of large fragments and the supernatant was transferred into new centrifuge tubes. In order to concentrate the trophozoites, tubes were centrifuged at 750x g for 5 min and the supernatant was discarded. The sediment was re-suspended with 0.65% sodium chloride solution and kept at room temperature for 30 min. Then the 0.65% sodium chloride solution was replaced by the new 0.65% sodium chloride solution. The centrifuge tubes were chilled in ice for 30 min and centrifuged at 2,000× g for 5 min, the supernatant was discarded and the trophozoites were collected from the pellet. The precipitated trophozoites were re-suspended with amphibian normal saline and were stored at 4 °C, the trophozoites sample was used for morphological and molecular analysis.

Samples were collected from March 2014 to May 2019 in 30 different study sites of 14 cities in China: Chuxiong, Dali, Honghe, Kunming, Xishuangbanna, Lincang and Pu'er of Yunnan Province; Liangshan, Mianzhu, Deyang and Chengdu of Sichuan Province, Longnan of Gansu Province; Hanzhong of Shaanxi Province; Shanghai. We followed the guidelines of The Animal Care and Use Committee of the American Society of Mammalogists for the use of wildlife in our research and used the recommended method for euthanizing targeted animals, which induce rapid unconsciousness and death without pain or distress (Sikes, 2016). All the experimental procedures and animal care were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences.

2.2. Morphological identification of G. agilis

All anuran amphibian samples were investigated. The trophozoite samples described above were dropped on slides. The slides were prepared with trophozoite supernatant in 0.65% sodium chloride solution and 20 mm square coverslips. All slides were checked before PCR and all coverslips were completely checked. All slides were examined under oil immersion by 40× and 100× HCX PL APO objectives on a Leica DM2500 microscope (Leica, Wetzlar, Germany). The images were captured by a Leica DFC450 C digital camera. Sample handling and photomicrographs of scanning electron microscope were processed at the Kunming Medical University (Kunming, China) using HITACHI 3700N (Tokyo, Japan).

2.3. Statistical analysis

Statistical analysis was performed using mid-P exact probability tests and 95% confidence interval (CI) value was calculated, differences were considered significant when p-values ≤ 0.05 were obtained (https://www.medcalc.org/).

2.4. DNA extraction, PCR amplification and sequencing

Girdia agilis genomic DNA was extracted from feces samples of tadpoles and frogs using the TIANamp Stolol DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The primers were designed according to the conserved sequences of these genes of *G. intestinalis* (50803 isolate) using PRIMER PREMIER program version 5.00 (Biosoft International) (Lalitha, 2004). These primers specific to both ends of the sequences of the two genes, *SSU rRNA* (GenBank accession numbers: M54878) and *beta-giardin* (GenBank accession numbers: KJ363393). The expected product lengths of the *SSU rRNA* and *beta-giardin* were 950 bp and 500 bp, respectively (primer pairs of *SSU rRNA*: '(680) AGCAGCCGCGGTAATTCC (697)' and '(1629) CCTTGTTA CGACTTCTCCTTCC (1608)'; *beta-giardin*: '(11) GCGAGGAGGTCAAGA AGTC (29)' and '(510) GAGCGTGTTGACGATCTTGT (491)'). Both feces DNA samples and trophozoite samples were amplified by PCR. The PCR reactions were set up in 25 μl 2× PCR Taq Plus MasterMix with dye (abm, Canada), 1 μM of each primer and 1–5 μl of DNA sample. Thermocycling conditions were as follows: 94 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, followed by 72 °C for 10 min. The PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Qiagen), and cloned into pMD-19T vectors using TaKaRa pMD-19T VectorCloning Kit (TaKaRa, Tokyo, Japan). The ligation products were transformed into DH5α chemically competent *E. coli*. Colony PCR was carried out with the vector-specific primers provided in the kit, and colonies were selected and Sanger-sequenced using vector-specific forward and reverse primers by TSINGKE Biological Technology (Kunming, China).

2.5. Molecular phylogenetic analysis

In order to perform the phylogenetic analysis, we sequenced, identified and retrieved the *SSU rRNA* and *beta-giardin* of *G. agilis* (GenBank accession numbers: MN227552 and MF185954). The other sequences used in the analysis were all retrieved from the GenBank database including *beta-giardin* of *G. intestinalis* Assemblage_A(KJ363393), *G. intestinalis* Assemblage_B(KJ363389), *G. intestinalis* Assemblage_D(KJ027418), *G. intestinalis* Assemblage_E (KJ363399), *G. intestinalis* Assemblage_F(KJ027424), *G. muris* (EF455599), *G. psittaci*(AB714977), *G. cricetidarum*(MF185953), *G. microti*(MF185955); and *SSU rRNA* of *G. muris*(X65063), *G. ardeae* (Z17210), *G. cricetidarum*(MF185957), *G. microti*(AF006677), *G. intestinalis*(dog)(AF199449), *G. intestinalis* Assemblage A(M54878), *G. intestinalis* Assemblage B(HG425134), *G. intestinalis* Assemblage E (HG425150). The number of isolates of *G. agilis* for each locus sequenced is as follows: 5 for *SSU rRNA* and 7 for *beta-giardin*. The maximum likelihood phylogenetic trees based on *SSU rRNA* and *beta-giardin* DNA sequences were reconstructed by using the PhyML 3.0 software (Guindon et al., 2010). The sequence data of the two loci are from 9 to 10 *Giardia* species or isolates. Multiple sequence alignments were performed with ClustalW 2.0 program (Larkin et al., 2007), and

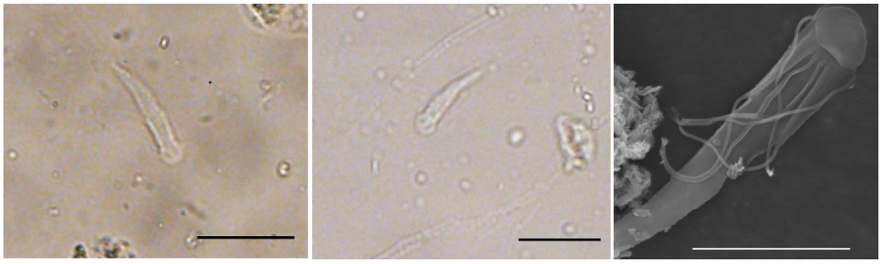

Fig. 1. Trophozoites of *G. agilis* A, B. The trophozoites of *G. agilis* under optical microscopy have a narrow and elongated body. Bar = 20 μm. **C.** Trophozoites of *G. agilis* under scanning electron microscope. Bar = 10 μm.

the alignments were visually inspected to eliminate poorly aligned positions. The best-fit DNA model used for reconstructing the maximum likelihood phylogeny was selected by the JModelTest 2 software (Posada, 2009; Darriba et al., 2012). The best-fit DNA model were F81 + G for *SSU rRNA* and TrN + I + G for *beta-giardin*. Trees were constructed using the PhyML 3.0 based on 14 taxa with 817 nt positions for *SSU rRNA* gene and 16 taxa with 335 nt positions for *beta-giardin* gene. Tree reliability was determined by using bootstrap analyses with 1000 replicates.

3. Results

3.1. Microscopy

All microscopic preparations were positive for the presence of *G. agilis* and trophozoites of *G. agilis* from positive samples were measured by microscopy. Trophozoites had an elongated body with lengths of 20–30 μm and widths of 4–5 μm (Fig. 1). But more accurate measurements could not be reached because *G. agilis* trophozoites are easily distorted morphologically during the purification from intestines.

3.2. Prevalence of G. agilis in 25 different anuran amphibian species

We investigated 25 anuran amphibian species in total. We investigated only adults in 19 species, only tadpoles in one species and both tadpoles and adults in five species. 195 of all the 463 (42.1%) investigated samples were tested to be positive for *G. agilis* using both morphological and molecular analysis. And the 195 positive samples were from nine species. The statistical prevalence of *G. agilis* in all these positive host species are shown in Table 1. Tadpole samples of all the six species were found to be positive. The 16 negative anuran amphibian species and tested samples were shown in Table 2, all negative samples were from adults. Though 16 species were tested to be negative, the prevalence of *G. agilis* in positive frog species showed no significant difference. The prevalence in the tadpoles of the six species was also not significantly different. In the five species samples with both adults and tadpoles, we found that the prevalence between adults and their tadpoles among each frog species were significantly different, and the total prevalence between the all adults and their tadpoles were also

Table 2
Tesed samples of the 16 *G. agilis* negative anuran amphibian species.

Sub-order	Family	Frogs	Stage	Tested Samples
Neobatrachia	Hylidae	*Hyla chinensis*	Frog	2
		Hyla annectans Jerdon		9
	Microhylidae	*Calluella yunnanensis*		24
		Microhyla ornata		9
	Rhacophoridaae	*Rhacophorus* sp.		1
		Polypedates chenfui		1
	Bufonidae	*Bufo gargarizans andrewsi*		2
		Duttaphrynus melanostictus		10
		Pelophylax plancyi		2
		Pelophylax pleuraden		15
		Rana grahami		8
	Ranidae	*Odorrana rodora*		5
		Quasipaa spinosa		18
		Paa yunnanensis		2
		Rana rugulosa		8
Mesobatrachia	Pelobatidae	*Oreolalax* sp.		8

Table 3
Prevalence of *G. agilis* in the 5 anuran amphibian species with both tadpoles and adults.

Frogs and Tadpoles		Positive/Tested Samples	Total Prevalence	95% CI
Adult	*Kaloula verrucosa*	4/19 (21%)	8.9%	2.48–21.24%
	Rana catesbeiana	0/3 (0%)		
	Amolops mantzorum	0/8 (0%)		
	Rana quadranus	0/11 (0%)		
	Odorrana margaratae	0/4 (0%)		
Tadpole	*Kaloula verrucosa*	86/92 (93.5%)	84.6%	77.78–89.99%
	Rana catesbeiana	7/22 (31.8%)		
	Amolops mantzorum	7/7 (100%)		
	Rana quadranus	16/16 (100%)		
	Odorrana margaratae	10/12 (83.3%)		

Table 1
Prevalence of *G. agilis* in the 9 positive anuran amphibian species.

Sub-order	Family	Positive Species	Stage	Positive/Tested (Prevalence)	95% CI
	Microhylidae	*Kaloula verrucosa*	Frog & Tadpole	104/126 (82.5%)	74.72–88.69%
	Rhacophoridaae	*Rhacophorus sp.*	Tadpole	22/22 (100%)	84.56–100.00%
	Ranidae	*Nidirana pleuraden*	Frog	12/66 (18.2%)	9.78–29.63%
Neobatrachia		*Rana chaochiaoensis*	Frog	22/29 (75.9%)	65.11–95.64%
		Amolops mantzorum	Tadpole	7/15 (46.7%)	21.29–73.44%
		Odorrana margaratae	Tadpole	10/19 (52.6%)	28.84–75.53%
		Rana catesbeiana	Tadpole	7/25 (28.0%)	12.07–49.39%
		Rana quadranus	Tadpole	16/27 (59.2%)	38.74–77.56%
Mesobatrachia	Pipidae	*Xenopus laevis*	Frog	2/2 (100%)	15.81–100.00%

Table 4
Prevalence of *G. agilis* in three different developmental stages of *Kaloula verrucosa* tadpoles.

Kaloula verrucosa Tadpole in Kunming	Prevalence (Positive/Tested Samples)	95% CI
Without legs	86.4% (19/22)	65.13–97.11%
With two legs	100% (10/10)	69.15–100.00%
With four legs	0% (0/4)	0.00–60.24%

significantly different (Table 3). Furthermore, in Xihuanyuan lake of Kunming (102.7°E, 25.0°N), we collected and tested *G. agilis* in different developmental stages of *Kaloula verrucosa* tadpoles, and found that the prevalence are as follows: tadpoles without legs (Gosner stages 26–30) – 86.4%; tadpoles with two legs (Gosner stages 31–40) – 100% and tadpoles with four legs (Gosner stages 41–46) – 0% (Table 4). The prevalence of *G. agilis* in different developmental stages of *Kaloula verrucosa* tadpoles from same area showed no significant differences between tadpoles without legs (Gosner stages 26–30) and tadpoles with two legs (Gosner stages 31–40), but the prevalence in both of them are significantly higher than the prevalence in tadpoles with four legs (Gosner stages 41–46), which was higher than adults.

3.3. Prevalence of G. agilis in anuran amphibians from different areas

The 463 frog samples were collected from 14 areas of five provinces in China (Fig. 2). For the information of these sampling areas, please see Supplementary Data Table S1. The prevalence of *G. agilis* in the 25 sampling frog species from these different areas is summarized, please see Supplementary Data Table S2. All samples from the same area were put together as calculating the prevalence in the area. We found that the prevalence among the positive frog species from areas with higher altitude (equal to or over 870m) showed no significant difference from each other, and the prevalence in the same positive frog species from these higher altitude areas also showed no significant difference (Table 5). On the contrary, the frog samples from the areas with lower altitude (lower than 870m) were all negative. Interestingly, all the positive samples came from these sampling places where the altitudes are more than 870m. For example, *Rana catesbeiana* is positive in Dali (1505m) and negative in Shanghai (5m), and *Amolops mantzorum* is positive in Deyang (870m) and negative in Chengdu (730m). And the total prevalence of areas in different altitude were significantly different: the total prevalence of areas with higher altitude (Higher than 870m, 46.53%, 95% CI 41.67–51.44%) were significantly higher than the areas with lower altitude (0.00%, 95% CI 0.00–8.04%).

3.4. Phylogenetic analysis of collected G. agilis samples

We sequenced a fragment of the *SSU rRNA* and *beta-giardin* genes of the collected *G. agilis* samples, respectively. Genetic distances between *G. agilis* and other *Giardia* species at the two loci are equal or greater than the differences between currently accepted *Giardia* species (Table 6). And *G. agilis* is most genetically close to *G. intestinalis* and *G. microti*. Phylogenetic analysis based on these fragments showed that *G. agilis* we collected is genetically distinct from all other *Giardia* species (Fig. 3A and Fig. 4A), and that all *G. agilis* we tested showed a small genetic distance, indicating they should be from same species despite the samples being collected from different hosts and areas (Figs. 3B and 4B).

4. Discussion

In the present study, the morphological characteristics were used to identify the *G. agilis* from all the frog samples, and the partial *SSU rRNA* and *beta-giardin* sequences were used as supplementary identification evidence. The anuran amphibian species were collected from 14 areas in China where natural environments are different from each other. We have tested *G. agilis* in 463 collected individuals from 25 anuran amphibian species consisting of adults and/or tadpoles, and found that nine species of them have positive individuals and the overall prevalence was 42.1% (195/463). The prevalence of the adults showed no significant difference in these frog positive species. All the tadpole

Fig. 2. Distribution of sampling positions All samples were collected from these 14 places of 5 provinces in China. The sizes of circles represent the sample sizes.

Table 5
Prevalence (95% CI) of *G. agilis* in the same species from different areas.

Area	*Kaloula verrucosa*	*Kaloula verrucosa* tadpoles	*Nidirana pleuraden*	*Amolops mantzorum*	*Rana chaochiaoensis*	*Rana catesbeiana*
Kunming	40.0% (12.16–73.76%)	92.0% (84.24–96.71%)	20.7% (11.18–33.36%)	–	–	0.0% (0%–21.80%)
Chuxiong	0.0% (0–33.63%)	–	0.0% (0–36.94%)	–	–	0.0% (0–97.50%)
Honghe	–	100% (82.35–100%)	–	–	–	–
Dali	–	–	–	–	–	100% (59.04%–100%)
Liangshan	–	–	–	0.0% (0–45.93%)	90.9% (58.71–99.77%)	–
Deyang	–	–	–	100% (59.04–100%)	50.0% (11.81–88.19%)	–
Mianzhu	–	–	–	–	100% (66.37%–100%)	–
Chengdu	–	–	–	0.0% (0–84.19%)	–	–
Shanghai	–	–	–	–	–	0.0% (0–84.19%)

Table 6
Genetic distances between *Giardia agilis* and other *Giardia* species (a) for the *beta-giardin* locus (b) for the *SSUrRNA* locus.

(a)

Species	1	2	3	4	5
1 *G. intestinalis*					
2 *G. cricetidarum*	0.104				
3 *G. muris*	0.105	0.033			
4 *G. psittaci*	0.138	0.151	0.166		
5 *G. microti*	0.100	0.112	0.109	0.129	
6 *G. agilis*	0.087	0.105	0.095	0.128	0.012

(b)

Species	1	2	3	4	5
1 *G. intestinalis*					
2 *G. muris*	0.769				
3 *G. ardeae*	0.681	0.586			
4 *G. microti*	0.582	0.572	0.659		
5 *G. cricetidarum*	0.561	0.779	0.584	0.685	
6 *G. agilis*	0.635	0.840	0.665	0.669	0.641

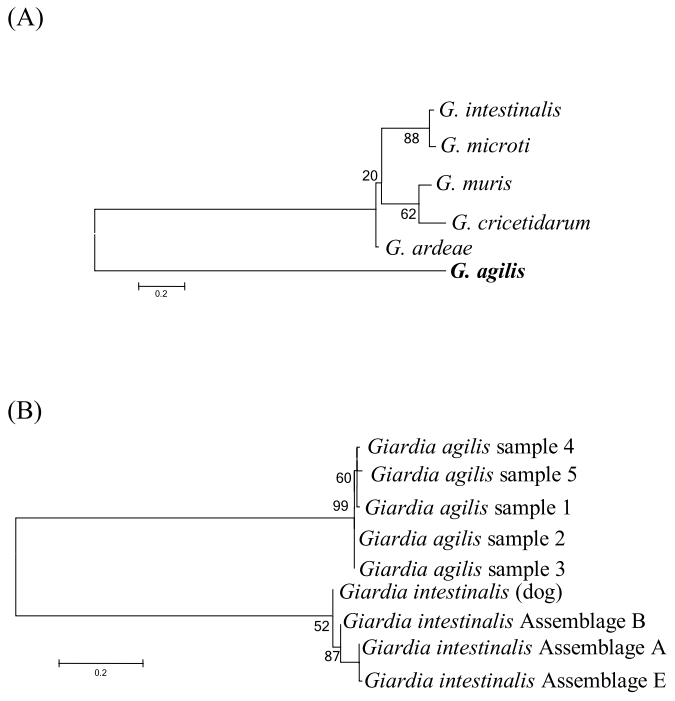

Fig. 3. Phylogenetic tree of *SSU rRNA* Phylogenetic tree of *SSU rRNA*, **A.** *G. agilis* is distinct from all *Giardia* species; **B.** all *G. agilis* we tested were from the same species.

samples from the six species were tested positive and the prevalence among them also showed no significant difference. On the other hand, the 25 frog species samples we tested were from seven anuran families, four of them: Microhylidae, Rhacophoridaae, Ranidae and Pipidae were tested to be positive, and three of them: Hylidae, Bufonidae and Pelobatidae were tested to be negative. Obviously, the positive species are not narrowly restricted but scatteredly distributed in four families that are not closely related to each other in the order Anuran (Hay et al., 1995). We further noticed that the prevalence in the same positive species from different high-altitude places also showed no significant difference. All these observations implied that *G. agilis* is probably able to infect all anuran amphibian species without species-bias, and some of the anuran amphibian species were tested to be negative might be due to their sample size was too small or their tadpole samples were absence. Prevalence of *G. agilis* show no preference in different anuran amphibians might imply that *G. agilis*, as a single species, has evolved some special parasitic adaptation or host-specific strategy so that it can almost indiscriminately infect all anuran amphibian species.

Interestingly, we found that there were significant differences of *G. agilis* prevalence between adults and their tadpoles. In five frog species we tested the prevalence of *G. agilis* both in their adults and tadpoles, and found that either in the single species or in the total species, the prevalence of *G. agilis* in tadpoles was found to be much higher than in adults. Furthermore, we found that the prevalence of *G. agilis* in different developmental stages of *Kaloula verrucosa* tadpoles from the same area was interesting. There were no significant differences between the tadpoles without legs (Gosner stages 26–30) and the tadpoles with two legs (Gosner stages 31–40), but the prevalence of them were both significantly higher than in the tadpoles with four legs (Gosner stages 41–46). This suggests that a turning point of prevalence of *G. agilis* was apparently exist in the period of front leg development of frogs, which might be related with the development of immune system of frogs. Because it is known that during the ontogeny of frogs, tadpoles exhibit changes in immune system cells transforming from larval-type to adult-type, especially changing sharply at the stage of tadpole tail degeneration (Izutsu, 2009), which is consistent with the “turning point of prevalence”. All other known *Giardia* species, they all infect animals without metamorphosis, such as *G. ardeae* and *G. psittaci* in birds, *G. microti* and *G. muris* in rodents, *G. intestinalis* in mammals (Lujan and Svard, 2011), *G. peramelis* in Australian bandicoots (Hillman et al., 2016) and *G. cricetidarum* in hamsters (Lyu et al., 2018), such a phenomenon has never been seen. Thus, this observation may imply that the transformations of immune system cells from larval-type to adult-type probably occur during the front legs growing. And prevalence of *G. agilis* in the developing tadpoles would decrease as the transformation begins and finally reaches a small level in the adults. Different from all the others known *Giardia* species, *G. agilis* has developed an appropriate parasitic adaptation strategy to the metamorphosis development of frogs.

It is also interesting that all the 195 positive samples were found to be collected from the areas where the altitudes are above 870m in our

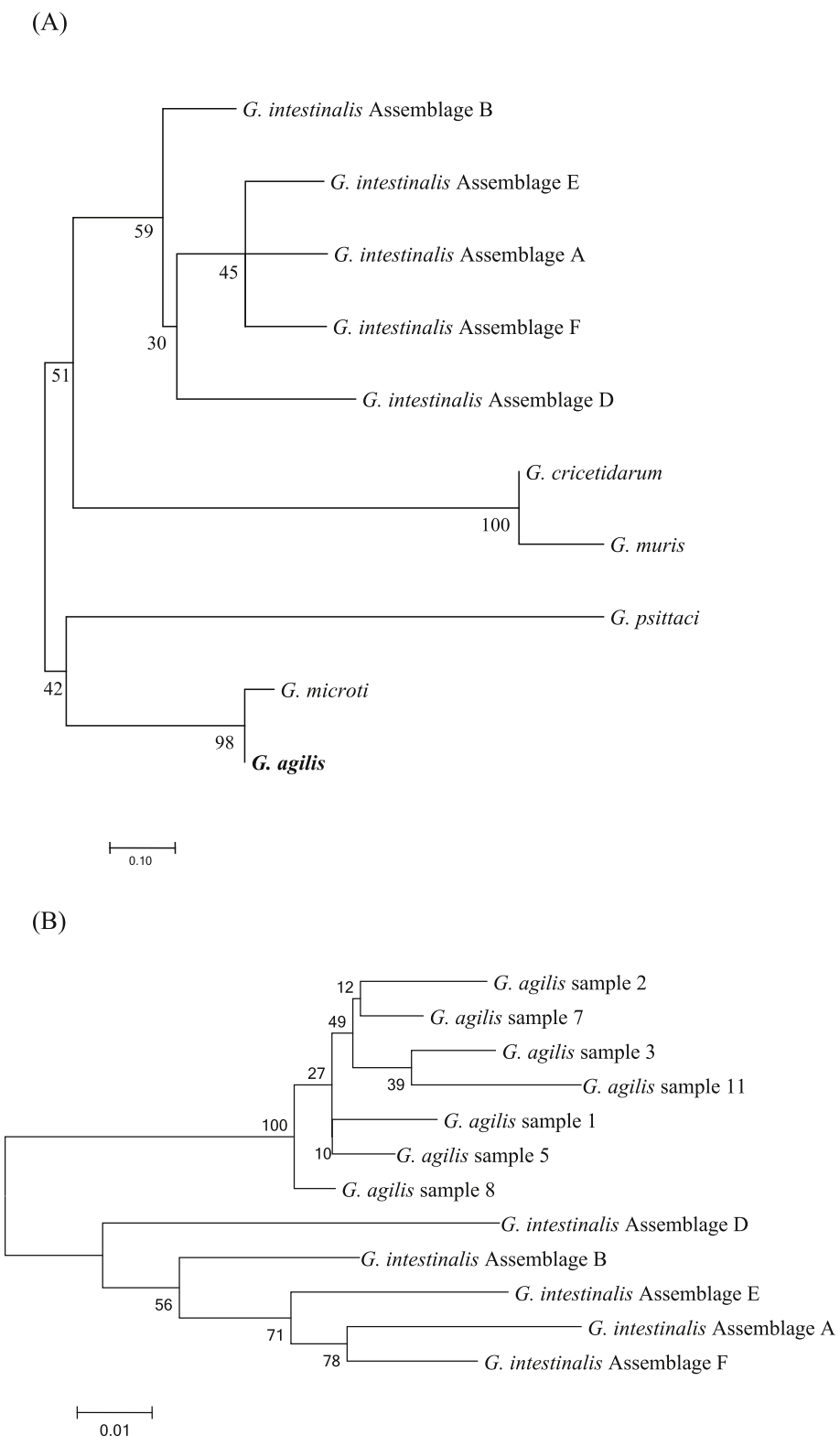

Fig. 4. Phylogenetic tree of *beta-giardin* Phylogenetic tree of *beta-giardin*, **A.** *G. agilis* is distinct from all *Giardia* species; **B.** all *G. agilis* we tested were from the same species.

investigation, including the same positive species from different areas. All these *G. agilis* positive frog species are not plateau specific. The average temperatures across areas where positive samples were collected are different (Ma et al., 2014). Furthermore, the temperatures often fluctuate dramatically during a short period of time at high altitude areas, and anuran amphibians would also change their body temperatures as they are poikilotherms. Therefore, different from other known *Giardia*, *G. agilis* parasitizes the frogs living in high altitude areas means that this species is always under the pressure of temperature changing. Compared to other known *Giardia* species, especially those

parasitizing homothermal animals, *G. agilis* in the poikilothermal animals has adapted to the dramatic temperature change. All these findings imply that *G. agilis* has evolved some special successful parasitism strategies for parasitizing the poikilothermal hosts with metamorphosis development – anuran amphibians, this adaptive mechanism is worthy of further study.

Our analysis at two different loci showed that *G. agilis* samples from different places showed a small genetic distance, and the genetic distances among different assemblages of *G. intestinalis* are even greater than among them. This means that not only that all these *Giardia* samples from different anuran amphibians must belong to the same species, but also that *G. agilis* might be under a greater selective pressure than *G. intestinalis,* which has limited the differentiation of this species. At least the remarkable immune system transformation of the hosts and the dramatic temperature change of its living environment mentioned above might be two extra resources of such great selective pressure. Therefore, *G. agilis* must have to evolve some special parasitic adaptation or host-specific strategy compared to other known *Giardia* species.

5. Conclusion

Our analysis at two different loci showed that *G. agilis* samples from different places showed little genetic distance, and the genetic distances among different assemblages of *G. intestinalis* are even greater than those among them. This means that not only all these *G. agilis* samples we tested in different anuran amphibians must belong to the same species, but also *G. agilis* might be under a greater selective pressure to show more conservative than *G. intestinalis*. At least the remarkable immune system transformation of the hosts and the dramatic temperature change of its living environment mentioned above might be two extra resources of such selective pressures. Therefore, *G. agilis* must have evolved some special parasitic adaptation or host-specific strategy compared to other known *Giardia* species.

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Declaration of competing interest

We confirmed that there are no known conflicts of interest associated with the publication and there has been no significant financial support for this study that could have influenced its outcome. We confirmed that our manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirmed the order of authors listed in the manuscript has been approved by all of us. We confirmed that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to the publication. We further confirmed any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval and that such approval was acknowledged within the manuscript. We confirmed that we have provide a current, correct email address which accessible by the Corresponding Author and which has been configured to accept email from (wenjf@mail.kiz.ac.cn).

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Appendix A. Supplementary data

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